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Development of a designed comparison method based on isotope dilution liquid chromatography–tandem mass spectrometry for determining plasma renin activity and its clinical assessment of renin activity stability in plasma†

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Plasma renin activity (PRA) is recommended as the first screening indicator for primary aldosteronism. Immunoassays and liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods have been developed for quantifying PRA, but the interchangeability across assays and laboratories was suboptimal, which predominantly related to the differences in the plasma incubation strategy. This study aims to establish and validate a designed comparison method based on LC-MS/MS. The sensitivity, matrix effect, precision, accuracy, and storage stability were validated according to the Clinical Laboratory Standard Institution (CLSI) C-62A guidelines. The plasma incubation procedure was optimized to achieve maximum PRA results. The short-term stability of PRA plasma was assessed at 4 °C and room temperature (RT) for specific time points. Differences from the baseline were calculated using a one-way analysis of variance. The designed comparison method for PRA measurement exhibits excellent performance characteristics. The results from the 2022 national external quality assessment scheme for PRA showed good consistency of the developed method with other LC-MS/MS methods (relative biases: –6.8% to 4.6%), which demonstrated the reliability of the established method. Two sets of generation buffers were optimized to maximize the renin activity. The acetate buffer was recommended to be used in laboratory practice due to better metrological sensitivity. PRA plasma is stable for one day at 4 °C and RT. In summary, a reliable, traceable, and reproducible LC-MS/MS method for determining PRA was well-established and validated. The recommended incubation protocol is hoped to reduce the discrepancy in Ang1 generation. The evaluated short-term stability for PRA plasma could provide flexibility in clinical practice.

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Introduction

Primary aldosteronism (PA) is one of the most common causes of secondary hypertension, characterized by significantly elevated plasma aldosterone and decreased plasma renin activity (PRA) levels, hypokalemia, and hypertension.^{1–3} A timely, precise diagnosis and treatment could effectively improve the prognosis and life quality of PA patients. The Endocrine Society⁴ has recommended the aldosterone-to-renin

ratio (ARR) as the first-line indicator for PA screening. Many studies suggested that ARR has a superior diagnosis efficacy to plasma potassium or aldosterone assays.^{3–5} PRA was measured as the denominator to calculate the ARR value, which plays an important role in the evaluation of disease conditions, especially in patients with slightly elevated plasma aldosterone levels.^{6,7}

Renin, an aspartate proteolytic enzyme released from the renal juxtaglomerular cells, substantially reflects PRA levels. Renin cleaves the endogenous substrate angiotensinogen (AGT) and produces angiotensin 1 (Ang1). PRA can be calculated as the amount of Ang1 produced per unit time during the incubation period, *i.e.*, [(the concentration of Ang1 after incubation minus before incubation)/incubation time], with the unit of ng mL^{–1} h^{–1}. This enzymatic assay can reflect the real condition and activity of individual renin–angiotensin systems since it takes into account the contributions of both enzyme (*i.e.*, renin) and substrate (*i.e.*, AGT) levels.⁸ The *in vitro* reaction of enzyme

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activity highly relied on an appropriate buffer system, since non-specific proteinase might hydrolyze Ang1 into other peptides (e.g., angiotensin 2 and angiotensin 1–7), and cause underestimation of PRA levels. Thus, proteinase inhibitors such as ethylenedinitrilotetraacetic acid (EDTA) and phenyl-methylsulfonylfluoride (PMSF) were added to the incubated plasma to avoid the degradation or transformation of Ang1.⁹ Incubation strategies consist of pH buffers, proteinase inhibitors, incubation time, *etc.*, which greatly influence the renin enzyme activity, and further produce variable results.

Accurate measurement of PRA was essential and troublesome. For advantages such as cost-effectiveness, high throughput, and practical simplicity, radioimmunoassay (RIA) and chemiluminescence immunoassay (CLIA) have been the mainstream assays for PRA determination for decades, but they also have several major drawbacks, e.g., cross-reactivity, antibody recognition sites, radioactive hazards, *etc.* Moreover, incubation procedures were extremely variable across different laboratories or manufacturers, since no (inter-) nationally recommended consensus is available. A comprehensive multicenter comparison was conducted among twelve European laboratories to determine the intra- and inter-laboratory reproducibility.⁸ A commercial diagnostic kit based on RIA for PRA assay was applied for the comparison. Citrate buffer (pH = 6) containing PMSF was added to each aliquot of plasma sample in duplicate, and then incubated at 37 °C and 4 °C for 90 min, respectively. Despite the use of uniform reagents and adherence to the instrument of the manufacturer, significant inter-laboratory coefficients of variation (CVs) were observed, particularly in low-concentration plasma (e.g., PRA: 0.14 ng mL⁻¹ h⁻¹; with a CV of 59.4%). The complex incubation details and inaccurate quantification may represent major obstacles to reproducibility and comparability among different assays and laboratories.

To improve the accuracy and reliability of PRA measurement, liquid chromatography–tandem mass spectrometry (LC-MS/MS) assays have been established.^{9–12} However, the lack of a harmonized incubation protocol hampered the interchangeability among different laboratories. According to the statistics from the 2021 National External Quality Assessment (EQA) Program organized by the National Center for Clinical Laboratories (NCCL, China, Available at: <https://www.nccl.org.cn/mainCn>), five EQA materials with a PRA concentration range of 1.88–13.63 ng mL⁻¹ h⁻¹ were provided for participating laboratories. Intra-assay CVs ranged from 23 to 66% (*n* = 12) for LC-MS/MS assays and 34 to 111% (*n* = 47) for CLIA assays. The mean biases between CLIA assays and LC-MS/MS methods ranged from –12.1% to 76.5%. Remarkable inconsistency existed among different assays and laboratories.

PRA assays are susceptible to environmental influences, such as temperature, cryoactivation of prorenin,¹³ plasma incubation conditions, non-specific proteinase degradation, *etc.* Therefore, it is necessary to achieve a consensus about plasma incubation for reducing the variations from the analytical process and further improving the harmonization status. In this study, we aim to establish a precise, reproducible, and reliable LC-MS/MS method for PRA measurement, and to evaluate the stability of PRA plasma.

Experimental

Chemicals and reagents

The Ang1 standard materials (AS-20627, 5 mg) with purity ≥95% and isotopic internal standard (IS) Ang1-[¹³C₆, ¹⁵N] were purchased from AnaSpec Inc. (USA). The standard reference material of Ang1 (SRM 998, 0.5 mg per vial) was obtained from the National Institute of Standards and Technology (NIST, USA); the purity is 99.9% ± 0.1%. The certified Ang1 purity value was assessed by HPLC and confirmed indirectly by nuclear magnetic resonance (within the reported uncertainty). Phenyl-methanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), zinc sulfate heptahydrate, bovine serum albumin (BSA), and ethylenedinitrilotetraacetic acid (EDTA) were purchased from Sigma-Aldrich (USA). Tris-base was obtained from Roche (Germany). Methanol, hexane, and formic acid were HPLC grade purchased from Fisher Scientific Co. (USA).

Analytical equipment and supplies

Ultra-pure deionized water (≥18.2 MΩ cm) was prepared using a Millipore-Q water purifier (Billerica, MA, USA). A 6500 plus triple quadrupole mass spectrometer (AB Sciex, USA) coupled with a Waters ACQUITY UPLC FI-I class system (Waters, USA) was used to perform the LC-MS/MS analysis. A Phenomenex Kinetex C18 column (2.6 μm, 100 mm × 2.1 mm) was purchased from Phenomenex (CA, USA). Oasis HLB cartridges used for solid-phase extraction (SPE) were obtained from Waters Corporation (USA).

Preparation of stock and working solutions

All standard and IS solutions were gravimetrically prepared. For the preparation of the Ang1 standard stock solution, deionized water containing 10% formic acid was used to dissolve the Ang1 standard materials. The IS Ang1-[¹³C₆, ¹⁵N] stock solution was prepared similarly. Ang1 working solution (17.6 ng g⁻¹) was prepared in 1% BSA buffer solution from stock solutions; this buffer solution consists of 0.1 mol L⁻¹ Tris in deionized water, adjusted to pH = 6 using glacial acetic acid. The IS working solution (10.7 ng g⁻¹) was diluted with deionized water containing 10% formic acid. Aliquots (1 mL) of stock and working solutions were kept in polypropylene Protein LoBind® tubes (Eppendorf) and frozen at –70 °C.

Plasma collection and sample preparation

Leftover EDTA plasma samples from outpatients or hospitalized patients who underwent PRA examination (unaffected or affected patients were both included) were collected from the endocrine laboratory of Beijing Hospital between September and December 2021 and were rapidly frozen at –70 °C. Two concentrations of quality controls (QCs) (*i.e.*, 3.8 and 10.1 ng mL⁻¹ h⁻¹) for PRA were prepared using pooled residual plasma and analyzed in each run. QCs and plasma samples were prepared by the following procedure. The collection of residual plasma had been approved by the Ethics Committee of Beijing Hospital.



Considering that the frozen plasma samples ought to be rapidly thawed at room temperature rather than in a refrigerator,^{13–15} plasma samples/calibrators/QCs were fully thawed at 37 °C for 3 min, to avoid cryoactivation and help to speed up the thawing process. The sample preparation consists of plasma incubation and extraction. Ang1 generation buffer consists of pH buffer and proteinase inhibitors. These proteinase inhibitors were prepared in 1 mol L⁻¹ sodium acetate aqueous solution (adjusted to pH = 5.6–5.7 using glacial acetic acid), with the final concentrations of PMSF, SBTI, and EDTA of 5 mmol L⁻¹, 200 mg L⁻¹, and 50 mmol L⁻¹, respectively. Particularly, it is better to add PMSF and SBTI to the inhibitor buffer before each use.

A bracketing calibration was used to quantify Ang1.¹⁶ Appropriate amounts of Ang1 standard and IS working solutions were exactly weighed using an electronic balance and fully mixed to produce the six-point bracketing calibrators with analyte-to-IS ratios ranging from 0.25 to 6. 60 µL of generation buffer was added to 200 µL of plasma, and the mixture was fully agitated and then incubated at 37 °C for 3 h. 5 µL of formic acid and 50 µL of IS solution were added to stop the incubation reaction. The mixture was equilibrated at 4 °C for 0.5 h.

Sample extraction comprised protein precipitation and solid-phase extraction (SPE). 800 µL of 0.1 mol L⁻¹ zinc sulfate in methanol/water (v/v, 50:50) was added to precipitate the

protein. After vortex mixing and centrifugation at 4 °C (12 000 rpm, 10 min), the supernatants were transferred to Oasis HLB cartridges which were preconditioned with methanol (1 mL) followed by water containing 5% formic acid (1 mL). The loaded cartridges were washed sequentially with 15% methanol/water (1 mL) and hexane (1 mL), then samples were eluted with methanol containing 1% formic acid (1.5 mL), and evaporated to dryness under nitrogen. The residuals were reconstituted with 60 µL of 20% methanol/water containing 2% formic acid. After centrifugation at 4 °C (12 000 rpm, 10 min), 50 µL of residues was used for LC-MS/MS analysis. The injection volume was 15 µL.

LC-MS/MS analysis

Chromatography was performed on a Kinetex C18 column which was maintained at 40 °C. The automatic sampler temperature is 10 °C. Mobile phase A contained 0.2% formic acid in deionized water and mobile phase B contained 0.2% formic acid in methanol. Initial conditions were 90:10 (v/v) mobile phase A: mobile phase B at a flow rate of 0.4 mL min⁻¹ with the following linear gradient steps: 0.5 min, 10% B; 1.5 min, 95% B; 3.5 min, 95% B; 3.6 min, 10% B and 5.0 min, 10% B. The total run time was 5 min.

The column eluate was injected into a 6500 plus triple quadrupole mass spectrometer (AB Sciex, USA) maintained in

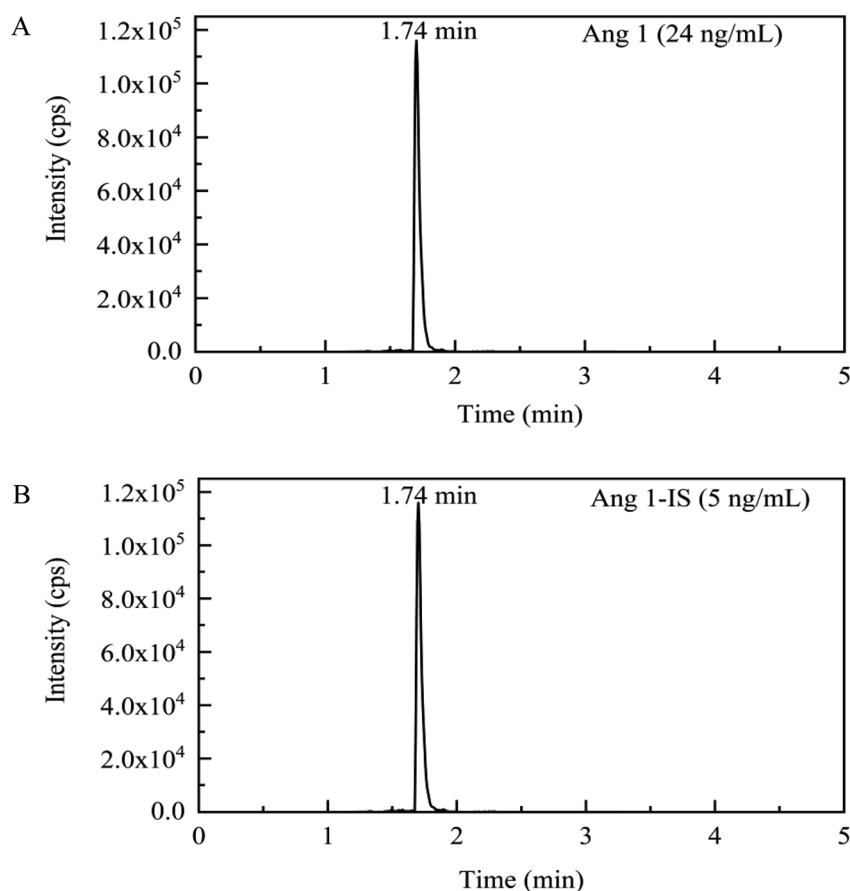


Fig. 1 A representative chromatogram of Ang1 and the IS extracted from a plasma sample.



electrospray positive ionization mode, with a source temperature of 450 °C and ionspray voltage of 5500 V. Multiple reaction monitoring (MRM) mode was used to analyze the mass transitions of Ang1. The most abundant transitions identified for Ang1 were m/z 433.2 \rightarrow 647.4 as a quantifier and 433.2 \rightarrow 619.4 as a qualifier. For Ang1-IS, m/z 437.6 \rightarrow 660.5 was a quantifier, and 437.6 \rightarrow 631.4 was a qualifier. Nitrogen was used as the curtain gas (CUR), nebulizer gas (GS1), auxiliary gas (GS2), and collision gas (CAD), and the pressures of the gases were set at 30, 40, 50, and 6 psi, respectively. Data are analyzed using Analyst 1.7 software (AB Sciex, USA). Typical chromatograms for Ang1 and Ang1-IS of an extracted plasma sample are shown in Fig. 1.

Quantitative method

Ang1 concentrations were calculated by comparing the analyte-to-IS ratios of the samples with those of the six-point calibrators. The mass fractions (ng g^{-1}) were converted to mass concentrations (ng mL^{-1}) by multiplying the density of the plasma measured with a density meter (DMA 4500 M, Anton Paar, Austria).

PRA was calculated using the following equation: $\text{PRA} = \frac{\text{Ang1}}{3} (\text{ng mL}^{-1} \text{ h}^{-1})$. Although it might be reasonable to subtract endogenous Ang1 (non-generated) from generated Ang1, many studies have proposed neglecting the blanks during measurements.^{12,13,15,17} Since the majority of endogenous Ang1 was usually below the limit of quantification, for the remaining measurable ones, endogenous Ang1 merely constitutes a minor proportion of generated Ang1 ranging from 5.6% to 8.5%, so they could exert little impact on the calculated results. Therefore, the calculation of PRA was simplified to the above equation.

Method validation

Absolute recovery, limit of quantification (LOQ), and limit of detection (LOD). The absolute recoveries were evaluated by comparing the IS peak areas obtained from the same amount of IS solutions added before and after sample extraction. LOQ and LOD were defined as the lowest concentration of analyte with signal-to-noise (S/N) ratios $\geq 10:1$ and $3:1$, respectively, and the $\text{CV} \leq 20\%$ for 20 injections. The standard solutions were processed according to the sample preparation procedure and were analyzed to evaluate the LOQ and LOD of the LC-MS/MS method.

Matrix effect. To evaluate the matrix effect and whether the IS could correct the potential matrix effect, the post-column infusion and matrix admixing experiment was adopted according to Clinical Laboratory Standard Institution (CLSI) documents C-62A¹⁸ and EP07.¹⁹ Firstly, the post-column infusion was adopted to qualitatively identify whether the ion suppression exists or not. 18.2 ng g^{-1} of Ang1 working solution after sample extraction was directly infused into the ion source at a constant flow rate of $20 \mu\text{L min}^{-1}$ via an integrated syringe pump and produced a stable background signal. The extracted matrix underwent chromatographic separation via the

autosampler and then mixed with the Ang1 working solution, and eventually, the mixture was injected for MS analysis.

Secondly, we prepared four different solutions to conduct the matrix admixing experiment: (X) the pure analyte/IS mixed standard solution; (Y) adding the same amount of analyte/IS mixed standard solution to the extracted matrix; (Z) equal volume of the extracted matrix only; (W) adding the same amount of IS solution to the extracted matrix. Therefore, the original matrix effect can be calculated using the following eqn (1):

$$\text{the original matrix effect (\%)} = \left(1 - \frac{A_Y}{A_X + A_Z}\right)\% \quad (1)$$

where A is the peak area of the analyte. The IS-calibrated matrix effect could be expressed as the following eqn (2):

$$\text{the IS-calibrated matrix effect (\%)} = \left(1 - \frac{R_Y}{R_X + R_W}\right)\% \quad (2)$$

where R is the analyte-to-IS area ratio. Plasma samples with different Ang1 levels were extracted by the above sample preparation procedure and then were adopted as the extracted matrix to evaluate the matrix effect.

Imprecision. To validate the imprecision of Ang1 and PRA measurement, four different concentrations of Ang1 plasma (no incubation) and three different concentrations of PRA plasma samples (performing incubation) were measured in five replicates in five runs on five different days according to CLSI EP15-A3.²⁰ The average of the five replicates was calculated as the final result. The intra-run, inter-run, and total imprecision were calculated according to one-way analysis of variance (ANOVA).

Accuracy and analytical recovery. Due to the lack of value-assigned reference materials (RMs) developed for Ang1 in the Joint Committee for Traceability in Laboratory Medicine (JCTLM) database, a spiking recovery experiment was used to evaluate the accuracy of the LC-MS/MS method. Two plasmas with different Ang1 concentrations (0.40 ng mL^{-1} and 18.61 ng mL^{-1}) were spiked with different amounts of Ang1 standard solution by gravimetric preparation to provide low-, medium-, and high levels of Ang1 samples. Samples were prepared by extraction and were measured in two replicates in three runs on three different days. The average results of three runs were calculated as the average recoveries. The national EQA materials (pooled plasma samples) were tested in three replicates per run for three days, and the results were compared with the target values which were calculated from the LC-MS/MS laboratory mean values ($n = 13$) using laboratory-developed tests (LDTs).

Optimization of plasma incubation buffer. Two sets of incubation buffers, *i.e.*, Tris and acetate, were adopted in previous studies.^{9,12} A series of concentrations of Tris, acetate, and proteinase inhibitors (PMSF and SBTI) were examined to explore the influence of different inhibitor buffers. The optimal concentrations for the buffers were chosen based on the calculated PRA results.

Stability of Ang1 working solution and plasma samples. The storage stability was evaluated according to CLSI documents C-



62A.¹⁸ Ang1 working solution was analyzed after storage under different conditions to evaluate the stability of standard calibrators. Samples were kept at 4 °C and −20 °C for 2 h, 6 h, 12 h, 24 h, 36 h, 48 h, and 72 h in two replicates. Samples kept at −70 °C were used as a baseline.

The collected plasma samples were thawed and mixed to prepare a plasma pool, and then aliquoted into 0.6 mL per vial. Samples were kept at 4 °C and RT (20 °C) from 0.5 h to 7 days to evaluate the stability of the PRA plasma during short-term storage. Two vials were placed under each storage condition and at each time point, each vial was measured in two replicates and the averages of replicates ($n = 4$) were calculated as the final results. Acetate buffer was adopted to perform plasma incubation in the subsequent application.

The influence of multiple freeze-thaw stability was also examined. Samples were thawed at RT for 1 h and frozen again at −70 °C. This action was performed in respective tubes once (FT1), twice (FT2) or three times (FT3) on different days. After the multiple freeze-thaw, all samples were frozen at −70 °C until further analysis.

Plasma frozen at −70 °C was used as a baseline. Samples under all storage conditions and at all time points were in two replicates, and the averages of replicates were calculated as the final results. Percentage changes of more than 10% from baseline concentration were considered as the stability threshold. The percentage changes (%) were calculated as $[(T_x - T_0)/T_0] \times 100\%$, where T_x is the result measured at a specific time point at a given temperature, and T_0 is the concentration of the baseline sample.

Livesey *et al.*²¹ suggested that they combined analytical imprecision and intra-individual biological variation as the total change limit (TCL), to evaluate the pre-analytical instability. To our knowledge, there is still no available meta-analysis or analytical performance specification updated for PRA. Hence, we chose 10% as the acceptable percentage change from the baseline concentration; this is generally used as a threshold for acceptable pre-analytical variability.^{22–24}

Data were analyzed using SPSS Statistics 26.0 (IBM Corp, USA). One-way ANOVA was used to assess the significance of differences. $p < 0.05$ was regarded as statistical significance.

Results and discussion

Absolute recovery, limit of quantification (LOQ), limit of detection (LOD), and linearity

The absolute recovery of the LC-MS/MS method ranged from 82% to 90%. The LOQ and LOD values of the LC-MS/MS method were 0.05 ng g^{−1} and 0.02 ng g^{−1} with a CV of 8.7% ($n = 20$). The average slope, intercept, and correlation relationship (R) with their 95% confidence interval (CI) obtained from 11 inconsecutive calibration curves used for analysis during one month were 0.067 (0.064 to 0.070), 0.069 (0.048 to 0.087), and 0.999 (0.999 to 1.000), respectively.

Matrix effect

The post-column infusion experiment verified that no apparent ion suppression or enhancement was observed near the retention time of the analyte or the IS peak. The matrix admixing experiment employed two plasma matrixes (low and high concentrations of PRA), and the results showed that the original matrix effect ranged from 10.3% to 41.0%, and the IS-calibrated matrix effect ranged from 2.6% to 5.2%, suggesting that the IS could efficiently compensate ion suppression for the LC-MS/MS method.

Imprecision

The imprecision of the LC-MS/MS method for Ang1 and PRA measurement is shown in Table 1. The intra-run, inter-run, and total CVs for Ang1 quantification were 1.12–4.05%, 1.14–2.59%, and 1.05–4.81%, respectively. The intra-run, inter-run, and total CVs for PRA (acetate buffer) were 2.78–3.39%, 3.64–8.58%, and 4.58–9.22%, respectively. The intra-run, inter-run, and total CVs for PRA (Tris buffer) were 3.47–5.08%, 4.30–6.11%, and 5.53–7.94%, respectively.

Analytical recovery and accuracy

A standard spiking and recovery experiment showed that the analytical recoveries of the LC-MS/MS method ranged from 95.8% to 106.9% (Table 2). The biases between the designed comparison method and target values of 2022 national EQA

Table 1 The imprecision validation of the LC-MS/MS method

		Mean	Intra-run CV, %	Inter-run CV, %	Total CV, %
Ang1 ^a	Sample 1	0.81	4.05	2.59	4.81
	Sample 2	2.19	2.04	1.14	2.33
	Sample 3	18.52	1.12	—	1.05
	Sample 4	31.06	1.71	2.07	2.68
PRA (acetate) ^b	Low plasma pool-1	3.01	2.78	3.64	4.58
	Medium plasma pool	5.43	3.39	8.58	9.22
	High plasma pool	31.29	2.99	7.87	8.42
PRA (Tris) ^b	Low plasma pool-2	2.44	4.08	5.90	7.18
	Medium plasma pool	4.61	3.47	4.30	5.53
	High plasma pool	27.78	5.08	6.11	7.94

^a The unit of Ang1 is ng mL^{−1}. ^b The unit of PRA (both for acetate and Tris buffers) is ng mL^{−1} h^{−1}; the medium and high plasma pools used in acetate and Tris buffers were the same, while the low plasma pools for the two buffers were different samples.



Table 2 The analytical recovery of the LC-MS/MS method

Spiked samples	Added concentrations, ng mL ⁻¹	Measured concentrations, ng mL ⁻¹	Recoveries, %	Mean recovery, %	Coefficients of variation, % (<i>n</i> = 6)
Sample 1		0.40			
Low	3.83	3.88	96.3–105.0	101.4	3.0
Medium	6.94	7.07	99.9–104.9	101.8	1.6
High	23.31	23.70	98.4–106.9	101.7	2.8
Sample 2		18.61			
Low	11.64	11.60	97.0–102.9	100.3	2.1
Medium	19.90	19.87	96.9–104.7	100.2	2.4
High	36.47	37.24	95.8–101.7	98.0	1.9

materials (with the concentrations ranging from 1.51 to 10.06 ng mL⁻¹ h⁻¹) were –6.8% to 4.6%, which were within the bias criterion of 15%²⁵ (1/2 total allowable error, *i.e.*, 30%, which referred to the acceptable limit of the 2022 national EQA scheme).

A reliable, robust, and traceable LC-MS/MS method was developed as the designed comparison method for Ang1 measurement. Ang1 is a basic polypeptide, and the addition of formic acid improves the solubility and stability of the standard material. 1% BSA solution and polypropylene Protein LoBind tubes help prevent peptide adsorption on the container surface. Ang1 was extracted and purified by protein precipitation and SPE, which produces a cleaner matrix and improves analytical reproducibility. We adopted the bracketing calibration with an isotopic ratio range of 0.25 to 6 and achieved satisfactory linearity for Ang1. All the standard calibrators and IS solutions were gravimetrically prepared. Differing from the common quantification method, which employed the volumetric method and linearity calibration with a wide concentration range, bracketing calibration allows laboratory technicians to adjust the amounts of samples and IS solutions weighed according to the actual concentration, especially for those beyond the isotopic ratio range; this could ensure that the area ratios fall within the measurable range to achieve superb linearity. No obvious matrix effect exists after the IS calibration. We adopted NIST SRM 998 as the available higher-order standard material to achieve the metrological traceability to the SI unit. The LC-MS/MS method exhibits excellent sensitivity, precision, and analytical recovery for Ang1 measurement, as well as good precision for the entire measuring procedure (*i.e.*, plasma incubation plus Ang1 determination). The results showed that the imprecision was ≤5% for Ang1 quantification and ≤10% for the PRA assay. It is of note that the analytical recovery experiment was performed on Ang1 quantification but did not include the plasma incubation procedure. Actually, we tried to add Ang1 standard solution to the plasma and then performed plasma incubation; the ultimately generated Ang1 was measured by the developed LC-MS/MS method, but the results showed lower Ang1 levels in spiked plasma compared with the non-spiked plasma (ESI Table 1†). One possible explanation could be that increased production caused negative feedback to the activity of the renin enzyme and lowered the reaction rate. The reliability of the established LC-MS/MS method was demonstrated through the 2022 national EQA scheme for PRA,

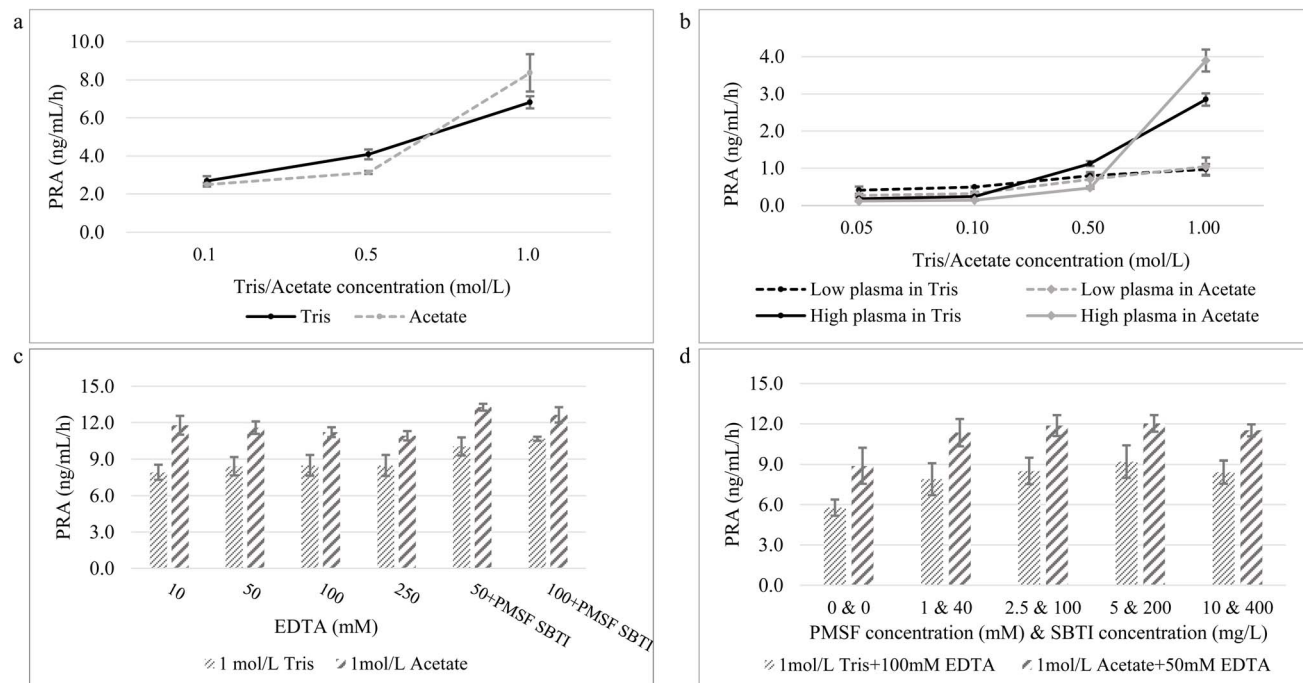
in which good consistency of the developed method with other LC-MS/MS methods (relative biases: –6.8% to 4.6%) was observed.

Optimization of plasma incubation buffers

The PRA levels elevated as the concentrations of Tris and acetate increased stepwise. When Tris and acetate concentrations reached 1 mol L⁻¹, the PRA reached the maximum value (Fig. 2a); this is consistent in both low and high PRA plasma (Fig. 2b). Next, the optimal EDTA concentrations in two buffers were evaluated. 50 mM EDTA in 1 mol L⁻¹ acetate buffer and 100 mM EDTA in 1 mol L⁻¹ Tris buffer (Fig. 2c) supplemented with PMSF and SBTi generated higher PRA. A combination of 5 mM PMSF and 200 mg L⁻¹ SBTi added to the optimized buffer would exert higher PRA results (Fig. 2d).

Little research was conducted on the influence of different buffers on Ang1 generation. We compared and optimized two mainstream buffer components^{9,12} to maximize the enzyme catalytic activity of renin and inhibit the non-specific degradation of Ang1. 1 mol L⁻¹ Tris or acetate was chosen as the pH buffer to achieve optimal activity of the renin enzyme. There are two other reasons to support this result. First, the commercially available Tris buffer is generally set at 1 mol L⁻¹, which is sufficient to meet the need to maintain pH constant. The higher concentration might cause incomplete dissolution and economic cost. Second, in previously published LC-MS/MS assays, 1 mol L⁻¹ Tris/acetate buffer was the most commonly used incubation buffer.^{12,15,17,26} For achieving the harmonization of the incubation strategy, the widely employed buffer setting is easier to adopt, and fewer changes would be required for laboratory professionals, and thus might facilitate the harmonization process. pH 5.6–5.7 was the favorable range for proteinase inhibition.²⁷ EDTA acts as a metalloprotease inhibitor to prevent the angiotensin-converting enzyme (ACE) from converting Ang1 to Ang2. In this study, 100 mmol L⁻¹ EDTA in 1 mol L⁻¹ Tris and 50 mmol L⁻¹ EDTA in 1 mol L⁻¹ acetate were adopted. But higher concentrations of EDTA (over 250 mmol L⁻¹) were not evaluated, because we found that during the solvent preparation, when EDTA concentrations were higher than 250 mmol L⁻¹, it is hard to achieve complete dissolution in Tris or acetate buffers despite an ultrasonic vibration over 30 min. In addition, the proteinase inhibitors (PMSF and SBTi) exert a positive influence on generated Ang1, with the





The optimized buffer parameters:

- (1) 1 mol/L Tris, 100 mmol/L EDTA, 5 mmol/L PMSF and 200 mg/L SBTI (pH=5.6-5.7)
- (2) 1 mol/L Acetate, 50 mmol/L EDTA, 5 mmol/L PMSF and 200 mg/L SBTI (pH=5.6-5.7)

Fig. 2 The optimization of the incubation buffer. (a) PRA generated from different concentrations of acetate or Tris; (b) different concentrations of acetate or Tris buffers incubated with low and high levels of PRA plasma; (c) PRA generated from 1 mol L⁻¹ acetate/Tris added with different concentrations of EDTA and/or PMSF + SBTI; (d) PRA generated from different concentrations of PMSF/SBTI.

appropriate concentrations of PMSF and SBTI being 5 mmol L⁻¹ and 200 mg L⁻¹, respectively (Fig. 2d); the add-on proteinase inhibitors might provide supplementary protection for proteinase degradation. The ultimate generation buffer settings are displayed in Fig. 2.

Two optimized generation buffers (*i.e.*, acetate and Tris) for plasma incubation were developed, however, the generated Ang1 significantly differed between the two buffers. Ang1 level was approximately 1.1 to 1.3-fold higher in acetate than in Tris buffer. In the present study, considering the quantitative sensitivity, we prefer to recommend acetate as the inhibitor buffer and use it in the subsequent determination. Furthermore, it is suggested that the correlation and comparability between the generation buffers deserve further studies.

The different generation buffers adopted in CLIA and LC-MS/MS methods were observed. Commercial CLIA diagnostic kits usually adopted EDTA, dimercapto-propanol, and 8-hydroxyquinoline sulfate as metalloproteinase inhibitors, while LC-MS/MS methods^{9,12,28} utilized EDTA, PMSF, and SBTI. The difference in inhibitors, as well as the cross-reactivity and sensitivity of CLIA, may together cause poor consistency (-12.1% to 76.5%) between CLIA and LC-MS/MS assays. This issue may be addressed by harmonizing the inhibitors between different assays to minimize the bias source from plasma incubation.

This study proposed a reliable and robust incubation procedure for PRA assay. When the validated incubation

strategy is introduced in LDT laboratories, the harmonization among LC-MS/MS assays would be improved greatly. After the application of the uniform protocol in LC-MS/MS laboratories, it is time to appeal for immunoassays to coordinate with this harmonized standard (*e.g.*, modifying their kits), and eventually improve the comparability across various laboratories and assays.

Stability of Ang1 working solution and plasma samples

The short-term stability experiment of the Ang1 working solution (Table 3) showed that the concentrations of Ang1 working solution stored at 4 °C and RT at different time points showed little change, ranging from 19.5 ng mL⁻¹ to 20.2 ng mL⁻¹ compared with the baseline concentration being 19.9 ng mL⁻¹ (percentage changes: -1.9% to 1.5%). Ang1 working solution is stable at 4 °C and RT for 3 days at least. The results of three freeze-thaw cycles for the Ang1 working solution were not significantly different from the baseline concentration (19.9 ng mL⁻¹), with the change percentage ranging from -0.6% to -0.3% (*p* > 0.05).

For plasma samples (Fig. 3 and Table 3), during storage at 4 °C, the PRA concentration increased after 3 days (from 8.3 ng mL⁻¹ h⁻¹ to 10.4 ng mL⁻¹ h⁻¹; +25.2%; *p* < 0.0001), and then increased by 29.6% after 7 days (from 8.3 ng mL⁻¹ h⁻¹ to 10.8 ng mL⁻¹ h⁻¹; *p* < 0.0001). During storage at RT, the PRA concentration increased after 3 days (from 9.6 ng mL⁻¹ h⁻¹ to 12.1 ng



Table 3 The short-term stability of Ang1 working solution and PRA plasma samples^a

Ang1 working solution, ng mL ⁻¹										
			Mean measured concentration (percentage difference, %), <i>n</i> = 4							
	<i>T</i> ₀	TCL (10%)	2 h	6 h	12 h	1 day	36 h	2 days	3 days	
4 °C	19.9	17.9–21.9	19.9 (+0.0%)	20.0 (+0.6%)	19.9 (+0.3%)	19.8 (−0.4%)	19.7 (−0.7%)	19.9 (+0.3%)	19.7 (−1.0%)	
RT	19.9	17.9–21.9	20.1 (+1.0%)	20.2 (+1.5%)	19.7 (−0.7%)	19.9 (+0.2%)	19.5 (−1.9%)	19.8 (−0.4%)	19.5 (−1.6%)	
Freeze–thaw			1 cycle	2 cycles	3 cycles					
	19.9	17.9–21.9	19.7 (−0.6%)	19.7 (−0.6%)	19.8 (−0.3%)					
PRA plasma, ng mL ⁻¹ h										
			Mean measured concentration (percentage difference, %), <i>n</i> = 4							
	<i>T</i> ₀	TCL (10%)	0.5 h	1 h	2 h	6 h	12 h	1 day	3 days	7 days
4 °C	8.3	7.5–9.1	8.8 (+6.5%)	8.5 (+2.7%)	8.7 (+5.2%)	8.0 (−3.3%)	8.6 (+3.3%)	8.5 (+2.2%)	10.4 (+25.2%)	10.8 (+29.6%)
RT	9.6	8.6–10.5	9.3 (−2.9%)	9.1 (−4.7%)	9.3 (−2.8%)	9.1 (−5.1%)	9.9 (+3.3%)	10.0 (+4.0%)	12.1 (+26.5%)	14.5 (+51.8%)
Freeze–thaw			1 cycle	2 cycles	3 cycles					
	8.8	7.9–9.6	9.3 (+6.0%)	8.5 (−2.7%)	8.5 (−2.4%)					

^a T₀, baseline concentration; TCL, total change limit.

mL⁻¹ h⁻¹; +26.5%; $p < 0.0001$), and rapidly increased more than half of the baseline concentration after 7 days (from 9.6 ng mL⁻¹ h⁻¹ to 14.5 ng mL⁻¹ h⁻¹; +51.8%; $p < 0.0001$). The results of three freeze–thaw cycles for PRA were not significantly different from the baseline concentration (8.8 ng mL⁻¹ h⁻¹), with the change percentage ranging from –2.7% to 6.0% ($p > 0.05$).

Several studies^{6,24} have evaluated the stability of DRC or PRA in whole blood collected into serum gel or EDTA plasma tubes before centrifugation. Locsei *et al.*⁶ proposed that when whole blood was kept at 4 °C for 2 h or at RT within 30 min before centrifugation, the PRA results remained unchanged. They also

recommended that –20 °C storage should not exceed 2 weeks, because PRA decreased by $9.4\% \pm 2.4\%$. Chakera *et al.*²⁴ found that DRC collected in EDTA plasma tubes dropped by over 10% after 6 h at RT (percentage change: –10.4%, 95% CI: –17.9% to –2.9%). However, few studies explore the short-term stability of post-centrifugation PRA plasma samples, and this knowledge is critical for EQA program providers and clinical laboratorians to handle EQA samples (plasma-matrixed pools for PRA measurement) throughout the analysis.

In this study, PRA plasma was observed to be stable for one day at 4 °C or RT; if the storage time is prolonged, the PRA

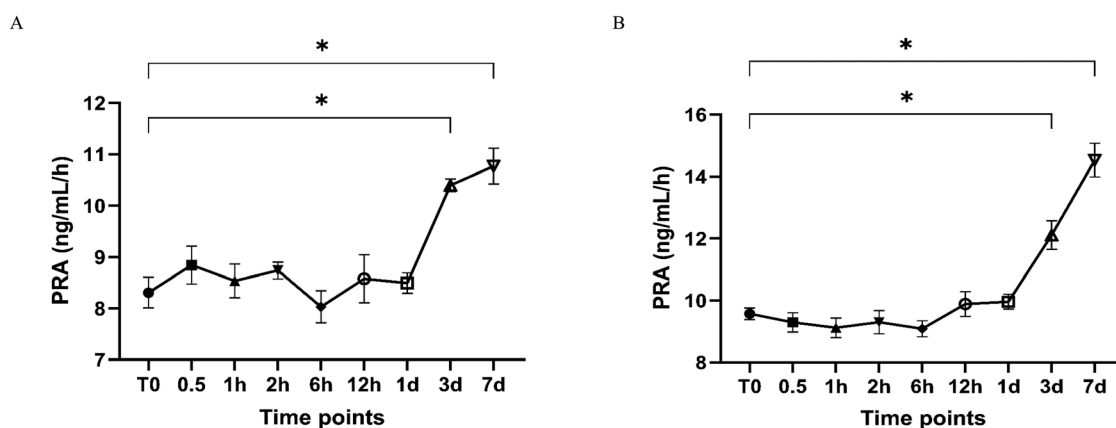
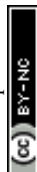


Fig. 3 The short-term stability of PRA plasma samples ($n = 4$) at 4 °C (A) and room temperature (B). The mean (standard deviation; error bars) concentrations of PRA (y-axis) vs. the storage time (x-axis). *Statistical significance ($p < 0.05$) vs. baseline concentrations (T₀).



results will significantly increase (>10%). This variation is probably attributed to the *ex vivo* renin catalysis activity or prorenin cryoactivation.⁶ Prorenin cryoactivation was expected to occur after the cooling stimulation lasting at least 6 h to 24 h,^{24,29} which is similar to our findings. This study provided more flexibility and convenience to laboratory technicians, especially when handling the EQA plasma materials.

We assessed the influence of freeze–thaw cycles and the results showed that PRA just changed slightly after three freeze–thaw cycles (−2.7% to 6.0%); this also provides confidence in the evaluation of the plasma stability study, since the inevitable freeze–thaw activities might cause little interference. In contrast, Hillebrand *et al.*³⁰ found that two or three freeze–thaw cycles would increase the PRA concentration significantly (+52.5% *vs.* baseline, $p = 0.0076$). These conflicting findings are probably due to different assay principles. The former study adopted an in-house RIA assay based on antigen–antibody binding, with an inter-assay CV of 12%; while the developed LC-MS/MS method showed better specificity and precision (total CV < 10%). Second, variable levels of PRA samples may exhibit changeable responses to prorenin cryoactivation or freeze–thaw stability,^{6,24} since low concentration ($1.2 \pm 0.4 \text{ ng mL}^{-1} \text{ h}^{-1}$) and high concentration ($8.8 \pm 0.1 \text{ ng mL}^{-1} \text{ h}^{-1}$) plasma were analyzed in the RIA assay and in this established method, respectively.

Therefore, this study may be a piece of the supplementary information for the clinical guideline,⁴ which suggests that for post-centrifugation plasma samples cannot be tested immediately, and short-term storage of one day at 4 °C or RT is acceptable; this would efficiently work against cryoactivation and avoid multiple freeze–thaw. The residual plasma should be immediately aliquoted and frozen in case any re-testing is required. Moreover, considering the poor stability at −20 °C, a more stringent storage strategy would be preferable (*e.g.*, −70 °C).^{6,31} More information about the long-term stability at −70 °C is needed.

One limitation of this study is that we did not evaluate the blank subtraction. Previous studies are in favor of neglecting the blanks, and potential benefits include lower sample volume and cheaper labor costs. The small proportion of blank to the generated Ang1 was considered to be quantitatively irrelevant.¹² However, a well-designed evaluation of the necessity of blank subtraction was needed in clinical practice, if necessary, in conjunction with its relevance to clinical decision-making. Additionally, we did not perform a detailed interference study because we only obtained the standard materials of Ang1 and Ang2; mass spectrometry analysis could distinguish the two compounds completely (MRM transitions at m/z 433.2 → 647.4 and m/z 524.0 → 263.2 for Ang1 and Ang2, respectively).

Conclusions

A robust, traceable, and reliable designed comparison method based on LC-MS/MS for measuring PRA was established and validated. The recommended incubation strategies might reduce the analytical variability of Ang1 generation, which currently limits the clinical application of this assay. Short-term

stability for one day at 4 °C and RT could provide flexibility in clinical practice.

Ethical approval

The collection of plasma was approved by the Ethics Committee of Beijing Hospital.

Informed consent

This study was approved for the exemption from informed consent.

Author contributions

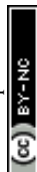
All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Conflicts of interest

The authors state no conflict of interest.

Abbreviations

PRA	Plasma renin activity
PA	Primary aldosteronism
CLIA	Chemiluminescence immunoassay
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
CLSI	Clinical Laboratory Standard Institution
CV	Coefficient variations
Ang1	Angiotensin 1
RIA	Radioimmunoassay
EQA	External Quality Assessment
NCCL	National Center for Clinical Laboratories
PMSF	Phenylmethanesulfonyl fluoride
SBTI	Soybean trypsin inhibitor
BSA	Bovine serum albumin
EDTA	Ethylenedinitrilotetraacetic acid
IS	Internal standard
CUR	Curtain gas
GS1	Nebulizer gas
GS2	Auxiliary gas
CAD	Collision gas
CRMs	Certified reference materials
ANOVA	One-way analysis of variance
JCTLM	Joint Committee for Traceability in Laboratory Medicine
LOQ	Limit of quantification
LOD	Limit of detection
S/N	Signal-to-noise ratio
TCL	Total change limit
LDTs	Laboratory-developed tests
RT	Room temperature



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